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13. ABSTRACT *(Maximum 200 Words)*

Ovarian cancer is the fourth leading cause of cancer-related death in U.S. women. This program project approaches the ovarian cancer problem by 1) establishing a human ovarian tissue and clinical data base core facility to support the proposed projects and future investigations, 2) identifying genes which are differentially expressed in ovarian cancers and thereby discovering biomarkers for early detection, 3) studying ovarian tumorigenesis in ovarian tissues obtained from germline *BRCA1* mutation carrier to better understand the interaction between mutational inactivation of *BRCA1*, the cellular caretaker gene and *p53*, the cellular gatekeeper gene, and 4) developing a genetically defined mouse model of epithelial ovarian cancer.

To date, the ovarian tissue core has banked over 100 surgical specimens and provided sufficient resources for the ongoing projects. Representational difference analysis was used to identify 46 genes overexpressed in normal ovarian epithelium and 14 genes overexpressed in ovarian cancer cells. The *BRCA1*-mutation associated ovarian tissues required to understand the functional interaction between of *p53* and *BRCA1* have been identified. And, a new viral construct carrying the cre recombinase under the control of the K18 promoter has been tested to establish its ability to mediate recombination in mouse ovarian epithelial cells.

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Genetic Definition and Phenotypic Determinants of Human Ovarian Carcinomas

Beth Y. Karlan, MD, Principal Investigator

INTRODUCTION:

It is estimated that 23,100 women will be diagnosed with ovarian cancer in the year 2000, and 14,000 women will succumb to this disease. For the first time, there has been a slight decline in the number of new ovarian cancer cases in the last year, and survival from ovarian cancer has been prolonged in recent years by improvements in surgery and chemotherapy. However, substantial progress towards ultimately eliminating ovarian cancer as a threat to women has been undermined by our ignorance about its etiology. Without additional insight into the genetic alterations that result in the clinical entity of ovarian carcinoma, we are left with empiric approaches to prevention, early detection and therapy. This program project is aimed at approaching the ovarian cancer problem by 1) establishing a human ovarian tissue and clinical data base core facility to allow the successful completion of the proposed projects and future studies aimed at understanding and eradicating ovarian cancer, 2) identifying genes which are differentially expressed in ovarian cancers and using this information to discover biomarkers for early detection, 3) studying ovarian tumorigenesis in "high risk" ovarian tissues obtained from carriers of germline *BRCA1* mutations to better understand the interaction between mutational inactivation of *BRCA1*, the cellular caretaker gene and *p53*, the cellular gatekeeper gene, and 4) developing a genetically defined mouse model of epithelial ovarian cancer which would be a vital tool for further studies of ovarian cancer etiology, prevention and therapy. This annual report prepared for the USAMRMC describes our progress towards achieving these goals during fiscal year 1.

BODY:

Ovarian Tissue and Clinical Database Core Facility
Beth Y. Karlan, MD, Principal Investigator

In the past, established epithelial ovarian cancer cell lines have been used to study the molecular pathophysiology of human ovarian cancer. These cell lines, however, are very distant from their original *in vivo* state. Following many generations of subculture, the subsequent genetic alterations and clonal selection biases that ensue make cell lines an imperfect experimental system for ovarian cancer. To address this problem, we have developed a human ovarian tissue resource and created a core facility to provide the necessary human ovarian tissues and cellular reagents required to successfully complete the three projects contained in this proposal.

TASK 1: Provide a continuing resource of normal and carcinomatous ovarian tissues from patients with benign gynecologic conditions and sporadic and familial ovarian cancer, respectively (months 1-36):

The Core Facility has provided all requisite ovarian tissues for the proposed projects. These have included: 15 snap frozen tumor specimens and 7 cell pellets from ovarian cancer ascites to Project 1 as well as multiple histopathology cut sections from prophylactic oophorectomy specimens removed from 7 *BRCA* mutation carriers to Project 2. In addition, during the first year of funding, the Core Facility has banked 103 surgical specimens, including 58 ovarian carcinomas and 45 benign ovaries. Of the 45 benign ovaries, at least 16 were donated from patients with a family history of ovarian and/or known *BRCA1* or *BRCA2* germline mutations. We also obtained matching germline DNA and serum samples from over 97% of patients donating surgical specimens. We have also 77 germline DNA samples from the Gilda Radner Ovarian Cancer Detection Program participants to our collaborators in Canada for *BRCA* mutation screening during this funding period. *BRCA1* and *BRCA2* mutation status has been recorded in the Core's Clinical Database.

We have met with difficulty in our efforts to establish ties with neighboring and international medical centers, in large part due to new regulations recently implemented by all participating Institutional Review Boards protecting the rights of human subjects involved in research. These new regulations, designed to provide better protection to human research subjects, include new, more detailed applications, separate Genetic Research applications, a new detailed consent form style and a training requirement for all investigators on the inclusion of human subjects in research. Drs Beth Karlan and Rae Lynn Baldwin have completed the required training and we have submitted new IRB applications and detailed consent forms to Cedars-Sinai and UCLA Medical Centers. When these applications and consent forms have Institutional approval, we will forward the new forms to the

USAMRMC for approval. In addition, there are new regulations for identifying and approaching patients for inclusion in human subject research which includes the donation of otherwise discarded surgical specimens. The new regulations state that the patient can only be identified and approached by their primary physician. We are actively recruiting the cooperation of all active gynecologic oncologists at Cedars-Sinai and UCLA Medical Centers in this endeavor. In addition, all participating/referring physicians must complete training in the inclusion of human subjects in research in order to be able to refer their patients. Despite these obstacles, we are making good progress establishing links with UCLA Medical Center that will allow us to routinely obtain ovarian specimens from their surgical suites as well.

TASK 2: Establish, characterize and maintain monolayer cultures of human ovarian epithelial cells, human ovarian stromal cells and human ovarian carcinomas according to established procedures (months 1-36).

We have provided the requisite ovarian epithelial cell cultures for the projects. Including: 10 CSOC cultures and 9 HOSE cultures to Project 1. In addition, of the 28 CSOC cultures established this past year, we have frozen cultures on 12. We have had a higher than usual attrition rate this past year due to fungal culture infections. These infections are likely due to construction in and around our tissue culture facility which has resulted in water leaks and other damage. The construction was to install an ambulatory care center on the floor above our tissue culture facility and was not under our control. We have taken all possible means to limit and eliminate these infections and have seen a dramatic decline in infection rate since the completion of the construction project. We have also established 18 HOSE cultures of which 9 have been cryopreserved and 6 lost and 17 ovarian stromal cultured of which 9 have been frozen.

TASK 3: Expand and maintain the clinical database to serve as an ongoing resource for translational studies (months 1-36).

We went "live" with the Ovarian Tissue and Clinical Database Core Facility electronic database in November of 1999 and became synchronous with current tissue procurement in January 2000. Clinical, epidemiologic and demographic data as well as specimen inventory information is now being entered as samples are collected and banked. We are actively formatting historical data on previously banked specimen for downloading and enhancing our reporting capabilities. In addition, we have been formulating plans for implementing data query and analysis software using the database platform.

Project #1, Molecular Biomarkers in Ovarian Cancer
David D. Chang, MD, PhD Principal Investigator

Cancer diagnosis is based on the detection of features that are unique to transformed cells. Each unique phenotype displayed in cancer cells must be accompanied by changes in gene expression. The genes that are differentially expressed in ovarian cancers compared to their normal counterparts therefore constitute logical candidates for molecular biomarkers for cancer detection. This application proposes to conduct a detailed analysis of gene expression differences underlying human ovarian carcinogenesis and use the information to develop biomarkers for ovarian cancer.

TASK 1: To clone genes that are differentially expressed in ovarian cancer and determine their expression profile (months 1-12).

The main objective of our project is to study the gene expression differences underlying human ovarian carcinogenesis. As proposed in Task 1 of the original application, we have conducted a representational difference analysis (RDA) using primary cultures of normal human ovarian surface epithelium (HOSE) and Cedars-Sinai ovarian carcinoma (CSOC). The rational for using cultured ovarian epithelial cells for gene expression analysis was based on the fact that the epithelial cells, which give rise to ~90% of ovarian cancer, constitute a very small fraction (<1%) of the total ovarian mass. We hypothesized that using primary cultures of normal and malignant ovarian epithelium for differential gene expression analysis would preferentially identify epithelial cell-specific genes. We have successfully identified 160 HOSE-specific and 95 CSOC-specific genes from our initial analysis, which employed HOSE and CSOC cultures obtained from two different patients. The expression of these cloned genes were surveyed in 5 additional HOSE and 10 additional CSOC cultures to identify 46 HOSE-specific and 14 CSOC-specific genes that exhibited at least 2.5-fold difference in expression level. Based on this encouraging result, we expanded our differential gene expression analysis into a gene expression profiling study. To date, we have examined gene expression profiles in 7 HOSE and 10 CSOC cultures using commercially available oligonucleotide-based microarray chips. We plan to examine additional HOSE and CSOC cultures, as well as primary tumors to further delineate gene expression profiles in ovarian cancer.

TASK 2: To characterize the protein coding information and subcellular localization of the differentially expressed genes (month 9-24).

In parallel to the above gene expression studies, we have started biochemical studies on selected genes as proposed in Task 2. To date we have focused on one gene that is significantly up-regulated in 8/11 CSOC cultures 5/6 ovarian tumors. This gene encodes a secreted protein of unknown

function. During Y02, we plan to generate antibodies to examine the expression of this gene in a large number of ovarian tumors.

Lastly, we have generated immortalized ovarian epithelial cell lines by engineering the expression of the catalytic subunit of human telomerase with or without co-expression of the E7 oncoprotein of human papillomavirus type 16 in primary cultures of normal or tumor-derived ovarian epithelial cells. In H02, we plan to carry out biochemical characterization of cloned cell lines and test the feasibility of using immortalized ovarian epithelial cell lines as an *in vitro* model for ovarian cancer will provide an opportunity to test the functions of cloned HOSE- and CSOC-specific genes to complement the ongoing gene expression profiling study.

TASK 3: To study the utility of differentially expressed genes as molecular biomarkers for ovarian cancer (months 21-36).

Not accomplished at this time.

Project #2, Interactions Between *BRCA1* and *P53* Mutations in Human Epithelial Ovarian Carcinogenesis
Mark D. Pegram, MD, Principal Investigator

Recent molecular epidemiologic studies have defined a role for the *BRCA1* tumor suppressor gene in familial breast and ovarian cancer syndromes. Using a full-length sequencing strategy, we have recently identified a high incidence (62/108) of *P53* tumor suppressor gene mutations in sporadic epithelial ovarian cancers. We hypothesize that familial ovarian cancer tumorigenesis caused by mutational inactivation and allelic loss of the cellular caretaker gene *BRCA1 requires* the mutational or functional inactivation of the cellular gatekeeper gene *P53* which controls cell cycle checkpoints and/or directs cells to undergo apoptosis. To test this hypothesis we have begun to collect and characterize *P53* expression levels and mutational status in ovarian cancers from a large cohort of known *BRCA1* mutation carriers.

TASK 1: Identification and histologic analysis of ovarian cancer specimens with sequence-verified germ line *BRCA1* mutations (months 1-36).

This task is relevant to Aim I of the proposal: to assess the frequency of *P53* gene mutations in epithelial ovarian cancers with known mutations of the *BRCA1* gene. During Y1 of the DOD project we have identified ~71 malignant ovarian tumors for which peripheral blood lymphocyte DNA is available and will be tested for *BRCA1* and *BRCA2* mutations. Based on the demographics of this

cohort, we expect ~40 patients will have *BRCA* gene mutations. We anticipate adding to this cohort prospectively by 10 – 15 new cases/year over the next two years.

TASK 2: Assessment of frequency of *P53* gene mutations in *BRCA1*-linked ovarian tumors identified in Task 1 above (months 6-36).

Task 2 is also relevant to Aim I of the proposal. In order to assess the frequency of *P53* mutation in *BRCA*-linked ovarian cancer, we first developed DNA sequencing reactions to study mutational frequency. This method is much more labor intensive and expensive compared to immunohistochemical (IHC) staining techniques, but it is the preferred method because of the high frequency of both false negative and false positive results from IHC. During Y1 we have obtained all of the necessary PCR primers for amplification of genomic DNA from ovarian tumor samples and we have established optimal conditions for each of the 10 different PCR reactions. We have confirmed the accuracy of this approach by sequencing *P53* from MDA-MB-231 cells which are known to harbor a specific *P53* mutation. We have also begun to identify *BRCA1* or *BRCA2* carriers in the Ovarian Tissue Core Facility database in order to obtain archival tumor material from these samples for sequence analysis. We had also proposed obtaining tumor tissues from collaborators in Israel (because of the high frequency of germline *BRCA* mutations in the local population). However, we have encountered great difficulty in procuring the samples needed because of new regulatory issues at the institutional and federal level regarding genetic testing. We have carefully insured that all subjects tested in our protocol remain anonymous and there are no patient identifiers in the database. However, more documentation must be provided to various regulatory authorities to complete this aspect of the project.

TASK 3: Assessment of MDM2 expression in *BRCA1*-linked and sporadic ovarian cancers using immunostaining techniques and compilation of *BRCA1* genotype data from all subjects (months 1-24).

Identification of established ovarian tumors in the existing tumor bank was proposed during Y1 for this task. Our current ovarian tumor bank contains ~800 samples, an ample number for conduct of the proposed analysis of MDM2 expression. This analysis is scheduled to begin in month 24 of the DOD funding, thus we are well ahead of the proposed schedule for this task.

TASK 4: To determine whether expression of wild-type *P53* in *P53*-mutant, *BRCA1*-mutant CSOC is sufficient to induce cell cycle arrest, induction of *P21* expression, and/or apoptosis (months 6-20).

This task addresses Aim III of the proposed research: to determine whether overexpression of wild-type *P53* is sufficient to induce cell cycle arrest/apoptosis in *BRCA1*-mutant ovarian cells. In this aim we will compare the biological effects of *P53* transduction in *BRCA1* mutant cell lines compared to cells without *BRCA1* mutation. The vector we have chosen for transfection studies is a replication deficient adenoviral vector containing the full-length human *P53* cDNA. This vector technology presents a technical problem which we have spent a large amount of time and effort to overcome. The problem we have identified is the fact that the adenovirus receptor (hCAR, human coxsackie and adenovirus receptor) has differential expression in our primary ovarian cell cultures. Therefore transduction efficiency by adenoviral vectors varies based on the expression level of the vector receptor. We have demonstrated for the first time that adenovirus receptor expression correlates with adenovirus transduction in ovarian cancer cells and we have recently completed an analysis of the chromosomal assignment of the hCAR gene (which maps to chromosome 21) and we have identified multiple pseudogenes for hCAR on chromosomes 15 and 18. With these data in hand, we will now be able to identify and *BRCA1* mutant cell line which has sufficient expression of hCAR so that we can complete the proposed transfection studies within the coming year. Our experimental observations on hCAR expression were an unexpected byproduct of the DOD funding, but potentially very important results in terms of patient selection for future clinical trials which might employ adenoviral vector technology for treatment or prevention of ovarian cancer.

TASK 5: To determine the frequency of *P53* alterations in "normal" ovaries from *BRCA1*-linked individuals who have undergone prophylactic oophorectomy (months 12-24).

This task addresses Aim II of the proposal: to determine the frequency of *P53* mutations in "normal" ovarian surface epithelium from *BRCA1*-affected individuals undergoing prophylactic oopherectomy. This aim was cited by reviewers as the most difficult to complete. However, we have made progress (ahead of the proposed schedule) during Y1 in identifying "normal" ovarian tissues from subjects with known *BRCA* mutations in germ line DNA. We have recently identified 8 subjects in the Ovarian Tissue Core Facility which meet criteria for study. 10 slides from each ovary have been cut and we are about to begin the first stage of this experiment which will consist of *P53* immunostaining to screen for evidence of *P53* mutation in these "normal" tissues. If evidence for such alteration can be identified at the tissue level, then we will conduct microdissection experiments followed by DNA extraction and PCR amplification for sequence analysis of *P53* gene. We anticipate IHC analysis of these cases in the coming months and sequence analysis within the

proposed timeline (months 12-24). We will identify additional specimens for prospective analysis in our database and from the tissue banks from our collaborators.

Project #3, Manipulation of Genes in the Ovarian Epithelium of Mice
Timothy F. Lane, PhD, Principal Investigator

Without models to test ideas about the initial stages of ovarian cancer, the task of identifying relevant markers and relevant targets for therapy becomes a daunting search for a needle in a haystack. One problem is the lack of a genetically defined animal model of epithelial ovarian cancer that can be used to test genes and gene pathways for their involvement during disease development. The strategy we proposed was to test the ability of wild type and modified adenovirus to deliver genes to normal ovarian epithelial cells *in vivo* with the idea that we could then use the cre-lox system to activate or delete genes of interest in a tissue specific fashion.

TASK 1: To establish the efficacy of Ad5-cre delivery to the ovarian epithelium (months 1-24).

The experiments specified in Task 1 of our Statement of work are progressing nicely. As specified in Task 1, used a recombinationally activated gene cassette that would allow the production of a β-galactosidase gene only in cells expressing cre recombinase, this cassette is referred to as RABE. Several experiments have been carried out in vitro showing that the components of the system work well in cultured primary epithelial cells, and this allowed us to move into work on RABE transgenic mice. To date, we have injected Ad-cre into the ovarian capsule of 4 RABE female mice. We have looked for expression of β-galactosidase at 8 hrs (2 mice) and 24 hrs (2 mice). β-galactosidase positive cells were identified only in the 24 hr time point and transduction appeared to be rather inefficient. We are currently producing more concentrated viral stocks in the hopes that poor infection rates can also be overcome with higher titers.

A complication was also quite evident. Adenoviral injections resulted in recruitment of a lymphoid infiltrate into sites of injection. We may request additional funds to document the cell types involved, but will likely switch to our proposed alternative strategy of transplanting the ovaries to avoid the need for exposure of animals to large amounts of virus.

We are also generating more mice to try the alternative strategy proposed for infecting the cells (ovarian transplants). This has been delayed because some of the mice became contaminated with a murine virus (MPV) and had to be destroyed. We are in the process of reestablishing our clean colony of RABE mice and hope to continue the experiments by mid October.

We have tested the K18 promoter and second promoter for K19 not originally described in the proposal. Both promoters drive expression in epithelial ovarian cells *in vitro* and in transgenic mice. We are in the process of creating viral stocks Ad-cre under the control of these promoters and will initiate *in vivo* experiments be the end of the year. Thus with respect to Task one, we are on schedule and will expand the scope to include a second epithelial promoter construct (K19).

TASK 2: To establish the ability of Ad5-cre delivery to delete genes from the ovarian epithelium (months 1-28).

The experiments specified in Task 2 are currently behind schedule due to a backlog in the availability of floxPTEN mice from Dr. Wu. As with our RAGE mice, the floxPTEN colony was dealt a set back from MPV infection which is now under control. We expect to be up and running with these longer-term studies by the end of the year.

TASK 3: To establish the ability of Ad5-cre delivery to activate over-expression of transforming oncogenes in the ovarian epithelium (months 12-36)

TASK 4: To establish the ability of Ad5-cre delivery to activate over-expression of dominant negative anti-oncogenes in the ovarian epithelium (months 24-36).

The experiments specified in Tasks 3-4 rely on development of plasmid vectors and transgenic mice in Task 3. The RA-her2 vector is currently near completion and will be tested in vitro ahead of schedule. We are also working with a new line of ES cells established from FVB mice which may allow us to do the initial transfections in a syngeneic mouse strain. If these cells prove to be effective, they could accelerate the project by several generations of mice.

KEY RESEARCH ACCOMPLISHMENTS:

Ovarian Tissue and Clinical Database Core Facility

- Collected and snap froze surgical specimen from 58 Ovarian cancer patients
- Collected and snap froze surgical specimen from 45 patients with benign ovarian of which 16 were from patients with family history of ovarian cancer or *BRCA1* or *BRCA2* heterozygotes
- Collected blood and isolated serum and genomic DNA from 97% of patients that donated surgical tissues
- Established primary cultures from 28 malignant ovarian tumors and 18 normal ovaries
- Cryopreserved primary cultures from 12 malignant ovarian tumors and 9 normal ovaries
- Provided all material requested to the 3 projects outlined in the Genetic Definition and Phenotypic Determinants of Human Ovarian Carcinomas Project
- Went live with the Ovarian Tissue and Clinical Database Core Facility electronic database

Project #1, Molecular Biomarkers in Ovarian Cancer

- Cloned genes that are differentially expressed in ovarian cancer cells using cDNA-RDA.
- Sequenced cloned DNA fragments to identify 160 HOSE and 95 CSOC specific genes.
- The cloned DNA fragments were used to fabricate a high density DNA arrays. These arrays were interrogated with cDNA probes from 15 different HOSE and CSOC cells to identify 46 HOSE-specific and 14 CSOC-specific genes that exhibited at least 2.5-fold difference in expression level.
- Constructed an ovarian epithelial cell cDNA library.
- Expanded the gene expression analysis by utilizing commercially available microarrays.* Established immortalized ovarian epithelial cell lines by transducing the catalytic subunit of telomerase (hTERT) and/or HPV-16 E7.*

*These accomplishments are in addition to the original goals.

Project #2, Interactions Between *BRCA1* and *P53* Mutations in Human Epithelial Ovarian Carcinogenesis

- Identified *BRCA1*-linked ovarian cancers in the Ovarian Tissue Core Facility
- Established optimal conditions for each of 10 PCR reactions required for amplification of genomic *P53* sequence. We have confirmed the validity of this approach by sequencing *P53* from MDA-MB-231 cells which are known to harbor a specific *P53* mutation.
- Identified a large cohort of both sporadic and *BRCA*-linked ovarian tumors suitable for MDM2 expression analysis.
- Discovered an association between expression of the human coxsackie and adenovirus receptor and adenoviral vector transduction efficiency in ovarian cell cultures.
- Elucidated anti-adenovirus antibodies as an inhibitor of adenoviral vector transduction in human malignant ovarian cancer ascites specimens.
- Procured the first 8 samples of "normal ovaries" from known *BRCA1* mutation carriers. We will seek evidence of *P53* gene mutation in these premalignant specimens.

Project #3, Manipulation of Genes in the Ovarian Epithelium of Mice

- Created a new construc that carries the cre recombinase under the control of the k18 promoter.
- Tested the ability of this construct to mediate recombination in mouse ovarian epithelial cells in culture.
- Generated Ad5-cre under the control of standard adenoviral promoter elements for pilot work *in vitro* and *in vivo*.

REPORTABLE OUTCOMES:

Ovarian Tissue and Clinical Database Core Facility

1. Established a well coordinated human tissue and serum repository for normal and malignant ovarian tissues and corresponding serum and germline DNA.

2. Routine development and preservation of primary cultures of normal and malignant human ovarian epithelial and stromal cells.

3. The Ovarian Cancer Laboratory and Clinical Database links laboratory specimens and results with patient demographic, epidemiologic and clinical data.

Project #1, Molecular Biomarkers in Ovarian Cancer

1. Ismail RS, Baldwin RL, Fang J, Browning D, Karlan BY, Gasson JC and Chang DC: Differential gene expression between normal and tumor-derived ovarian epithelial cells. In Press: Cancer Research, 12/01/00.

2. Generated immortalized ovarian epithelial cell lines by engineering the expression of the human telemorase catalytic subunit.

Project #2, Interactions Between *BRCA1* and *P53* Mutations in Human Epithelial Ovarian Carcinogenesis

1. Elkas JC, Pegram MD, Nielsen L, Tseng Y, Baldwin RL, Slamon DJ and Karlan BY: Immunoglobulins in malignant ascites inhibit adenoviral infection of tumor cells: Implications for adenoviral gene therapy. (Submitted)

2. Buller RE, Slamon D, Runnebaum IB, Horowitz JA, Buekers T, Salko T, Petrauskas S, Shahin M, Kreienberg R, Karlan B, Pegram M: A phase I/II trial of rAD/*p53* (SCH 58500) gene replacement in recurrent ovarian cancer. (Submitted)

Project #3, Manipulation of Genes in the Ovarian Epithelium of Mice

None thus far.

CONCLUSIONS:

This program project is a multidisciplinary collaboration aimed at elucidating genetic alterations that contribute to human ovarian carcinoma with an eye towards identifying useful targets for early ovarian cancer detection and prevention. Towards these ends, the **ovarian tissue core facility** has banked over 100 surgical specimens, including 58 ovarian carcinomas and 45 benign ovaries, of which approximately one third are from women with a family history of ovarian cancer of or a known *BRCA* mutation. Furthermore, the Core's tissue resources are linked to clinical, demographic, and epidemiologic data that allows us to make clinical correlations with our laboratory findings. In **Project 1**, representational difference analysis was used to identify 46 genes significantly overexpressed in normal ovarian epithelium and 14 specific genes overexpressed in ovarian cancer cells. Using these subtractive cloning techniques as well as a chip based expression profiling approach, we have successfully identified target genes that could be developed into biomarkers for ovarian cancer. In addition, the newly established immortalized ovarian epithelial cell lines will be used to study the functions of the genes that are differentially expressed in cancer-derived ovarian epithelial cells. **Project 2** has established the necessary techniques and identified the *BRCA1* mutation associated ovarian tissues required to understand the functional interaction and contribution of *p53* and *BRCA1* to ovarian epithelial transformation. In addition, as a byproduct of these studies, we discovered that adenoviral gene transfer may only be efficiently accomplished in ovarian cells which express a gene called hCAR (human coxsackie and adenovirus receptor). This observation may have far reaching implications for patients undergoing gene therapy for ovarian cancer using adenoviral vectors. **Project 3** has focused efforts at creating the necessary viral constructs for the proposed experiments aimed at establishing a murine human ovarian cancer model. A new construct carrying the cre recombinase under the control of the K18 promoter has been tested to establish its ability to mediate recombination in mouse ovarian epithelial cells.

"So what?" In order to reduce the unacceptably high mortality rate associated with ovarian cancer, diagnostic modalities which can reliably detect early stage ovarian cancer and preventative strategies to diminish the number of new cases must be discovered. This Program Project has undertaken a multi-faceted approach to the ovarian cancer problem. Using the human ovarian specimens and clinical correlates provided by the core facility, new genes will be identified in Project 1 to serve as targets for detection, prevention, and/or therapy; functional interactions between important known genes, *BRCA1* and *p53*, will be elucidated in Project 2 and shed light on the molecular etiology of ovarian cancer; and a murine animal model to test these findings and others in vivo will be established in Project 3. At the conclusion of this program project, we will be closer to our goal of rationale rather than empiric approaches to ovarian cancer prevention, early detection, and therapy.

REFERENCES:

None

APPENDICES

1. Ismail RS, Baldwin RL, Fang J, Browning D, Karlan BY, Gasson JC and Chang DC: Differential gene expression between normal and tumor-derived ovarian epithelial cells. In Press: Cancer Research, 12/01/00.

Differential Gene Expression between Normal and Tumor-Derived Ovarian Epithelial Cells

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Running Title: Gene Expression in Ovarian Epithelial Cells

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ABSTRACT

The majority of ovarian tumors arise from the transformation of the ovarian surface epithelial cells, a single layer of cells surrounding the ovary. To identify genes that may contribute to the malignant phenotype of ovarian cancers, cDNA representational difference analysis (cDNA-RDA) was used to compare expressed genes in primary cultures of normal human ovarian surface epithelium (HOSE) and ovarian tumors derived epithelial cells from the Cedars-Sinai Ovarian Cancer repository (CSOC). A total of 255 differentially expressed genes were identified, of which 160 and 95 were specifically expressed in HOSE and CSOC cells, respectively. Using cDNA array hybridization the expression profiles of the genes identified by cDNA-RDA were examined in additional 5 HOSE and 10 CSOC lines. The comparison of average signal of each gene revealed 44 HOSE-specific and 16 CSOC-specific genes that exhibited at least a 2.5-fold difference in expression. A large number of genes identified in this study encode membrane-associated or secreted proteins, and hence, may be useful as targets in the development of serum-based diagnostic marker for ovarian cancer. Very few genes associated with protein synthesis or metabolism were identified in this study, reflecting the lack of observable differences in phenotypic or growth characteristics between HOSE and CSOC cells. Northern blot analysis on a subset of these genes demonstrated comparable levels of gene expression as observed in the cDNA array hybridization.

INTRODUCTION

Ovarian cancer is the fourth leading cause of cancer-related deaths in the United States (1). Each year approximately 23,000 women will be diagnosed with this disease, and close to 14,000 will succumb to it. A significant factor contributing to the high mortality rate of ovarian cancer is the relatively asymptomatic progression of this disease. As a consequence, most patients are diagnosed with advanced (stage III/IV) disease when widespread intraperitoneal metastases are already present (2). Greater than 90% of ovarian malignancies arise from the transformation of the ovarian surface epithelium, a single continuous layer of epithelial cells surrounding the ovary.

There are estimated to be 20,000 genes expressed in a typical cell, and approximately 1% of those are differentially expressed in cancerous versus normal cells (3). A limited number of genes has been found to have elevated or depressed levels of expression in ovarian cancers when compared to normal tissue (4-8). As in other neoplasm, it is generally accepted that both activation of oncogenes and inactivation of tumor suppressor genes are involved in the etiology of ovarian carcinomas. Brca1, Brca2 and p53 mutations are associated with the development and progression of ovarian cancer (9-11). As these proteins are involved in the maintenance of genomic integrity, loss of their functions is thought to result in the accumulation of genetic mutations, leading to extensive changes in gene expression (12-15). Comparison between gene expression profiles of normal ovarian epithelial cells and ovarian tumors could identify candidate genes for biological markers of cellular transformation, possibly leading to earlier detection and new therapy.

Because ovarian epithelial cells represent a small proportion of the total cells found in the normal ovary, it is difficult to obtain primary material that is free of contaminating ovarian stromal cells in large enough quantities to conduct comparative gene expression studies. However, ovarian epithelial cells can be isolated and expanded in culture for approximately 15 passages (16, 17). The ability to culture human ovarian epithelial cells from both normal ovaries and ovarian carcinomas provides an opportunity to study differential gene expression between relatively pure populations of normal versus tumor-derived epithelial cells. This type of comparison minimizes gene expression differences that reflect the presence of non-epithelial cells, such as stromal or germ cells of normal ovaries and host-derived immune cells in ovarian tumors.

We utilized cDNA representational differences analysis (cDNA-RDA) to identify a set of genes that are differentially expressed between primary cultures of normal and tumor-derived ovarian epithelial cells. cDNA-RDA was subsequently combined with cDNA filter array hybridization to identify a subset of genes that are aberrantly expressed in a large number of malignant ovarian epithelial cells. Direct gene expression profiles were obtained by northern blot analysis on 4 differentially expressed genes to confirm the cDNA array analysis.

MATERIALS AND METHODS

Primary ovarian epithelial cell cultures. The conditions for growing normal human ovarian surface epithelial (HOSE) cells *in vitro* were modifications of the method described by Auersperg et al. (18). Briefly, normal ovarian tissue was obtained from the operating room from consenting donors, and placed in 199:MCDB 105 (1:1) medium (Sigma, St. Louis, MO) containing 10% FCS, 200 u/ml penicillin and 200 µg/ml streptomycin. Epithelial cells were microdissected or scraped from the ovarian surface. The epithelial explants were placed in culture medium and allowed to attach and proliferate. Once the epithelial cells reached confluency, the explants were removed and the cells were subcultured. Cedars-Sinai Ovarian Cancer (CSOC) cultures were established from ovarian carcinomas in a similar manner. Fresh tumor tissue was finely minced with scissors and allowed to attach to culture dishes in McCoy's 5A medium (Gibco BRL, Grand Island, NY) supplemented with 10% FCS and Pen/Strep. The epithelial nature of HOSE and CSOC cultures was verified by immunohistochemical analysis with antibodies against cytokeratin (AE1/AE3; Roche, Indianapolis, IN), vimentin (clone v9; Roche), and factor VIII (Factor VIIIC; Calbiochem), as previously described (19). p53 status of cultures was determined by immunostaining with Ab-6 antibody (Roche).

Cell lines. TfxH, an SV40 Large T antigen-immortalized HOSE cell line, was grown in 199:MCDB 105 (1:1) medium containing 10% FCS (17). Ovarian carcinoma-derived cell lines, Caov-3 and Sk-OV-3 (American Type Culture Collection, Rockville, MD), were grown in the presence of 10% FCS in DMEM or McCoy's 5A medium, respectively.

Cloning of differentially expressed genes using cDNA-RDA. cDNA-RDA was used to compare gene expression between two HOSE and two CSOC cultures (20). Total RNA was prepared from each culture, using RNA STAT-60 reagent (Tel-Test, Inc., Friendswood, TX). Messenger RNA was purified from 120 μg of total RNA using Oligotex mRNA columns (Qiagen, Chatsworth, CA), and used for cDNA synthesis. cDNA from HOSE and CSOC samples was digested with Dpn II and PCR-amplified following the addition of RDA adapters. Subtractive hybridization was performed in 2.5 μl of 3X EEP buffer (10 mM EPPS[N-(2-hydroxyethyl) piperazine-N'-3-propanesulfonic acid], 1 mM EDTA, 1M NaCl and 10% polyethylene glycol) for 21 h at 67° C. Two rounds of subtraction were performed in both directions, using tester to driver ratios of 1:100 and 1:500 for the first and second rounds, respectively. Finally, the cDNA-RDA products were digested with Dpn II and cloned into the BamHI site of pBluescript KS^+ (Stratagene, La Jolla, CA).

Amplification of individual cDNAs. Individual bacterial transformants were isolated into 96-well microtiter plates containing 100 μl of LB-ampicillin (100 μg/ml) and incubated overnight at 37° C. Using a 96-well replicating tool (V&P Scientific, San Diego, CA), bacterial culture was transferred into 96-well thermowell plates (Costar, Cambridge, MA) to inoculate 50-μl PCR reactions containing 250 μM dNTPs, 1 ng/μl SK (GGCCGCTCTAGAACTAGTGGATC) and KS (TGATATCGAATTCCTGCAGCCCG) primer each, and 0.03 u/μl Taq polymerase (Qiagen). Amplification was carried out for 35 cycles (94° C for 45 s, 68° C for 45 s, 72° C for 1 min), with a final 10-min extension at 72° C. The average size of the PCR-amplified fragments was ~500 base pairs (bp).

Identification of non-redundant clones. The PCR-amplified inserts were spotted onto 7.8 x 12.3-cm Hybond N+ membrane (Amersham Life Sciences, Arlington Heights, IL), using a 96-well replicating tool. Redundant clones were eliminated by back hybridization, as previously described (21). The DNA sequences of non-redundant clones were determined using a commercially available sequencing kit (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit, Perkin-Elmer, Foster City, CA). The nucleotide sequences were analyzed using the BLASTN program and the GenBank database.

cDNA filter hybridization of arrayed non-redundant clones. Non-redundant cDNA-RDA fragments were organized into 96-well plates and subsequently arrayed in duplicate on nylon membranes. cDNA array hybridization was carried out as previously described (21). To generate probes, ~50 μg of total RNA from each of 5 HOSE and 10 CSOC cultures were polyA-selected and used to synthesize cDNA. One-fifth of the total cDNA obtained was labeled with [^{32}P]-α-dCTP, using a random primer labeling kit (Prime-it II, Stratagene). Hybridization signal was quantified on a phosphorimager (Molecular Dynamics), using the ImageQuaNT software package.

Standardization of quantitative data. The signal intensity per pixel within each square of the grid was calculated and corrected for the background. Each filter was also spotted with actin, tubulin, glyceraldehyde-3-phosphate dehyrdrogenase (G3PDH) and translation elongation factor (EF-Tu) cDNAs. These genes function in housekeeping activities in the cell, and display little variability in expression between normal and transformed cells (22). EF-Tu, which showed the least variability in mRNA expression between all the samples, was used as the internal standard in this study. The signal of each background-corrected spot was determined relative to

average background-corrected signal for EF-Tu within a given filter, and averages of HOSE and CSOC standardized signal were compared for each of the 864 dots on the filter. In this study, only those with differences of greater than 2.5-fold were considered to be differentially expressed.

Northern blot analysis. Total RNA (5 μg) was separated on 0.9% agarose formaldehyde gels and transferred to Hybond-N+ nylon membranes. Filters were hybridized with ^{32}P-cDNA probes corresponding to the differentially expressed genes, as previously described (21). The filters were then stripped and re-hybridized with ^{32}P-labeled EF-Tu cDNA to control for mRNA loading.

RESULTS

Ovarian epithelial cells can be expanded in vitro. Ovarian epithelial cells tend to assume atypical fibroblast-like morphology and a dual epithelio-mesenchymal phenotype that is characterized by the expression of both keratin (an epithelial marker) and vimentin (a mesenchymal marker) in culture (16). Cultures containing endothelial cells, identified by Factor VIII staining, and ovarian stromal cells, characterized by the lack of cytokeratin staining and low levels of vimentin staining, were excluded in this study. The staining patterns for HOSE and CSOC cells used in the cDNA-RDA and cDNA filter array hybridization are presented in Table 1.

There are no established molecular criteria to distinguish HOSE from CSOC cells. Therefore we relied on cytogenetic studies to confirm that HOSE and CSOC cultures represent normal and malignant ovarian epithelial cells, respectively. Both HOSE cultures used in the cDNA-RDA contained normal female diploid cells; however, the cells from both CSOC cultures displayed an abnormal karyotype (Table 1). Sixty percent of CSOC 817 cells exhibited a loss of the X chromosome, which is commonly associated with ovarian carcinomas (23-25). Rearrangements in chromosomes 12 and 18 were also seen in CSOC 817 cells. Ten percent of CSOC 826 cells contained a small chromosome that may be isochromosome 21. A gain in small regions of chromosome 2 was also observed in CSOC 826 cells, which is consistent with a previous study showing frequent gains in discrete regions of chromosome 2 in some ovarian cancer cells (26). These abnormal karyotype profiles of CSOC 817 and CSOC 826 are consistent with the malignant origins of these cultures.

cDNA-RDA was used to identify differentially expressed genes in ovarian cancer cells. A total of 255 non-redundant genes were identified after two rounds of subtractive hybridization. Of these, 95 were preferentially expressed in CSOC cells and 160 in HOSE cells. We then used cDNA array hybridization to identify a subset of genes that were differentially expressed in a larger cohort of HOSE and CSOC cultures. Duplicate filters were spotted with genes identified by cDNA-RDA and hybridized with ^{32}P-labeled cDNA probes derived from additional 5 HOSE and 10 CSOC cultures. Forty-four HOSE-specific and 16 CSOC-specific genes displayed greater than 2.5-fold difference in expression (Tables 2 and 3). Example of cDNA filter arrays probed with ^{32}P-labeled cDNAs from HOSE 224 or CSOC 869 are shown in Fig 1. cDNA array hybridization easily identified connexin 43 (Cx43) and osteoblast-specific factor 2 (OSF-2) as HOSE- and CSOC-specific genes, respectively. The hybridization signal intensity of Doc-1, which was cloned as a HOSE-specific gene in cDNA-RDA, on the other hand, did not vary significantly between these 2 HOSE and CSOC cultures. The expression levels of EF-Tu, a housekeeping gene shown to be expressed at a constant level in normal and cancer cells (22), remained unchanged in HOSE 224 and CSOC 869 cells.

To assess the predictive value of cDNA array hybridization analysis, 4 differentially expressed genes were chosen at random and evaluated by northern blot analysis. OSF-2 was cloned as a CSOC-specific gene by cDNA-RDA, while the other three, keratin 19 (KRT19), Cx43, and stanniocalcin (STC), had been identified as HOSE-specific genes. The expression levels of OSF-2 were greatly elevated in the 2 CSOC samples used for cDNA-RDA, as well as in 7 of the 10 CSOC samples used in array hybridization (Fig 2). On the other hand, all three HOSE-specific genes were expressed at lower levels in CSOC cells (Fig 2). For example, there was an overall decrease in Cx43 mRNA expression in CSOC samples compared to HOSE

samples. STC and KRT19 were down-regulated in all but 2 CSOC samples. Interestingly, there was very little correlation in the expression of these genes in established ovarian cell lines. Specifically OSF-2 was not overexpressed in neither of the two established ovarian cancer cell lines, Sk-Ov-3 and Caov-3. In addition Cx43 which was expressed at varying level in both HOSE and CSOC cultures were not detected in neither ovarian cancer cell lines or an SV40 Large T-antigen immortalized TfxH cell line.

The majority of genes identified by cDNA-RDA was found to be differentially expressed by less than 2.5-fold in cDNA array hybridization, and eliminated from further consideration. To ensure that this arbitrary cut-off level did not eliminate any genes of interest, we performed northern analysis on 2 cDNA clones displaying an expression level difference of less than 2.5-fold (Fig 3). Northern analysis demonstrated that Doc-1, which was identified as a HOSE-specific gene in our study, as well as in a previous study (4), was expressed at higher levels in HOSE samples. However, its expression was variable, accounting for the less than 2.5-fold difference in cDNA array hybridization, which used average signal intensity in data analysis. Glia-derived nexin (GDN), a CSOC-specific gene, was up-regulated in both the CSOC 826 and CSOC 817 cells used in the initial cDNA-RDA analysis. In the additional HOSE and CSOC samples, however, GDN was expressed at a variable level in both normal and tumor-derived epithelial cells.

OSF-2 is overexpressed in both ovarian tumors and in vitro expanded CSOC cells. We next studied the expression of OSF-2 in ovarian tumor samples from which the CSOC cells used in this study were derived. Total RNA was isolated from the quick frozen tumor samples corresponding to 6 of the 12 CSOC cultures used in this study. Northern analysis revealed expression of OSF-2 in all but one tumor sample (T949) (Fig 4). The OSF-2 expression was low in the CSOC culture (C824), derived from the same tumor (see Fig 3A). In T1040, the level of

OSF-2 expression was lower than the level observed in the corresponding C871, but was easily detectable. Overall there was a high degree of concordance in the OSF-2 expression in tumors and the corresponding CSOC cultures, indicating that the observed overexpression of OSF-2 in CSOC cultures is not a consequence of in vitro expansion of tumor-derived epithelial cells.

Discussion

Identifying genes that are differentially expressed in ovarian tumors when compared to their normal counterpart is a challenging issue because of the scarcity of the normal ovarian epithelial cells. Ovarian surface epithelia from which ovarian cancers originate represent a minute cellular component of the ovary, compared to the more abundantly present stromal cells and germ cells. We utilized in vitro expanded ovarian epithelial cells derived from either normal ovaries or ovarian tumors to identify 60 genes that are either up- or down-regulated in ovarian cancer cells. In this study only the cancer cells derived from papillary serous histology tumors, which is considered to be the most common histological type of ovarian cancer, were included to limit the complexity of gene expression analysis.

Because of the variability in signal intensities in array hybridization analysis, we focused our attention on genes displaying more than 2.5 fold difference in the expression level. While the reliance on cDNA array hybridization with this arbitrary cut-off may have eliminated some differentially expressed genes from our analysis, it was highly effective in identifying genes that display a consistent pattern of expression differences in a large number of CSOC samples. Among the subset of genes displaying an expression level difference of greater than 2.5-fold, there was strong qualitative correlation between cDNA array hybridization data and northern analysis. Northern analysis confirmed KRT19, Cx43 and STC as HOSE-specific genes, and OSF-2 as a CSOC-specific gene. Two genes, Doc-1 and GDN, with expression level differences of less than 2.5-fold in cDNA array hybridization, on the other hand, displayed only a moderate difference (e.g., GDN) or a high sample-to-sample variability (e.g., Doc-1) in northern analyses.

The gene expression patterns seen in HOSE and CSOC cells were not consistent with those seen with TfxH cells, an immortalized HOSE cell line, or Caov-3 and Sk-OV-3, two ovarian carcinoma-derived cell lines,. As these cell lines were originally derived from normal epithelial or tumor-derived epithelial cells, similar to HOSE and CSOC cells (31), our results illustrate the divergence of gene expression that can occur as the result of long-term in vitro manipulation of these cells. Although cell lines provide a relatively simple model to examine gene expression in ovarian cancer-derived cells, our findings emphasize that the use of HOSE and CSOC cultures represents a better model system of normal and cancerous ovarian tissues in comparative gene expression analysis.

The use of cultured ovarian epithelial cells is not without concerns. Ovarian tumors frequently are histologically inhomogeneous (2). There are reports in the literature of loss of tumor markers associated with continuous tissue culture of some xenograft lines (27-30). Despite the primary tumors containing areas of differentiated cells, in each instance, a selection of a poorly differentiated subpopulation had occurred during the propagation of these lines. While we have utilized primary cultures to avoid a selection bias inherent in any long term cultures, we cannot formally excluded the possibility that our cultured CSOC cells represent sub-population of cancer cells present in the original tumor or in vitro expansion conditions may have modified gene expression. In the case of OSF-2, there was a high degree of concordance in its expression in tumors and their corresponding CSOC cells, indicating that despite in vitro expansion process, the expression of this particular gene is preserved in the cultured cells.

The extent of gene expression differences between HOSE and CSOC cultures is not known. Numerous genes identified in this study, including Doc-1, have previously been shown to

be differentially expressed in ovarian carcinomas (4-8, 26, 31). Comparing our data to previous studies on ovarian cancer-related genes revealed only a minor degree of overlap, indicating that the extent of gene expression differences far exceeds the number of genes identified in this or other previous studies. Genes associated with protein synthesis or mitochondrial metabolism are frequently identified in differential gene expression analysis of tumor tissues when compared to the normal tissue, and have been attributed to differences in proliferative and metabolic rates (8, 32). The absence of such genes in our analysis probably reflects the use of HOSE and CSOC cells, which are morphologically indistinguishable and display similar growth characteristics. This finding further emphasizes the utility of HOSE cells as well-matched controls in comparative gene analysis to identify aberrant gene expression in CSOC cells.

Several of the genes identified in this study are noteworthy. OSF-2 was originally reported as a transforming growth factor-beta (TGF-β)-inducible protein secreted in the extracellular matrix of the periosteum (33). The potential significance of OSF-2 in ovarian cancer is illustrated by the high degree of OSF-2 overexpression observed in both ovarian tumors and cultured CSOC cells. OSF-2 overexpression in CSOC cells may be a consequence of inappropriate TGF-β signaling that can be seen in some CSOC cells (19, 34). While the function of OSF-2 is not known, OSF-2 or a related protein βig-H3 is believed to function as a matrix protein that promotes cell attachment (33, 35). One possibility is that OSF-2 expression may facilitate intraperitoneal spread of cancer cells, which leads to a significant morbidity and mortality in women with ovarian cancer. STC is expressed at high levels in organs derived from the mullerian duct (36), and therefore, the loss of STC expression in CSOC cells may reflect cellular de-differentiation.. Cx43 and Cx40, which encode gap junction proteins, were cloned as HOSE-specific genes. Decreased gap junction communication and loss of Cx43 expression have been

reported in ovarian cancer (37), and may be related to the loss of epithelial cell features in cancer cells, as well as decreased cellular communication that is seen in many types of cancers.

In conclusion, the availability of primary epithelial cultures from both normal and malignant ovaries has enabled the identification of 60 differentially expressed genes, using a combination of subtractive hybridization and cDNA array expression studies. Despite the lack of observable phenotypic or growth differences between HOSE and CSOC cells, reciprocal expression of OSF-2 with STC, KRT19 and Cx43 was seen, reflecting differences in these cells at the molecular level. A large number of genes identified in this study encode transmembrane or secreted proteins (see Tables 2 and 3) that may be present in serum and could be used as marker for ovarian cancer. In addition, the genes identified in this study may provide clues to the cellular changes responsible for metastatic progression of ovarian cancer.

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Figure Legends

Figure 1. Analysis of cloned cDNA-RDA fragments by cDNA filter array hybridization. cDNA filter array hybridization was used to screen 255 differentially expressed gene fragments obtained after two rounds of cDNA-RDA. PCR-amplified fragments were spotted in a 96-well format onto nylon filter membranes. Duplicate filters were hybridized with random primed ^{32}P-labeled cDNA probes derived from HOSE culture H224 (A) and CSOC culture C869 (B). The positions of connexin 43 (Cx43) (1), Doc-1 (2), osteoblast specific factor-2 (OSF-2) (3), and translation elongation factor EF-Tu (4) are boxed. The differences in the signal intensities of Cx43 and OSF-2 in H224 and C869 can be visually appreciated. In contrast, the signal intensities of Doc-1 in these two samples were not significantly different. The expression levels of EF-Tu, which remained relatively constant in H224 and C869, were used to standardize the hybridization signal.

Figure 2. Northern blot analysis of gene that were differentially expressed by >2.5-fold. RNA prepared from 7 HOSE and 12 CSOC cells used in cDNA-RDA and cDNA array analysis were hybridized with ^{32}P-labeled probes of 4 randomly chosen genes that were differentially expressed by greater than 2.5 fold in cDNA array analysis. Osteoblast specific factor-2 (OSF-2) was cloned as a CSOC-specific gene (A), while stanniocalcin (STC), connexin 43 (Cx43), and keratin 19 (KRT19) were cloned as HOSE-specific genes (B). In cDNA array analysis, the average signal intensity of OSF-2 was 65-fold greater in CSOC cells, compared to HOSE cells. STC, Cx43, and KRT19 were preferentially expressed in HOSE cells by 7-fold, 2.6-fold, and 7-fold, respectively. The filters were stripped and reprobed with translation elongation factor (EF-Tu) to ensure integrity of RNA, and to determine RNA loading differences.

Figure 3. Northern blot analysis of genes that were differentially expressed by <2.5-fold.
RNA prepared from 7 HOSE and 12 CSOC cells used in cDNA-RDA and cDNA array analysis were hybridized with Doc-1 or glia-derived nexin (GDN) probes. Doc-1 and GDN were cloned as HOSE- and CSOC-specific genes, respectively. In cDNA array analysis, the average signal intensities the Doc-1 was 1.6-fold greater in HOSE cells, while the signal for GDN was almost even in CSOC and HOSE cells. The same filter membrane was stripped and reprobed with translation elongation factor (EF-Tu) to ensure integrity of RNA, and to determine RNA loading differences.

Figure 4. OSF-2 expression in ovarian tumors.
RNA prepared from ovarian tumors were hybridized for osteoblast specific factor-2 (OSF-2). The 6 tumors used in this analysis represent original ovarian tumors from which 6 of the 10 CSOC cultures used in cDNA array analysis were derived. Ethidium bromide staining of ribosomal RNA is provided below to control for the integrity and amount of total RNA (5 μg) loaded in each lane.

REFERENCES

1. Landis, S. H., Murray, T., Bolden, S., and Wingo, P. A. Cancer statistics, 1998. CA: Cancer J. Clin *48*: 6-29, 1998.

2. Cannistra SA. Cancer of the ovary. New Engl. J. Med., *329*: 1550-1559, 1993.

3. Zhang, L., Zhou, W., Velculescu, V. E., Kern, S. E., Hruban, R. H., Hamilton, S. R., Vogelstein, B., and Kinzler, K. W. Gene expression profiles in normal and cancer cells. Science, *276*: 1268-1272, 1997.

4. Mok, S. C., Wong, K. K., Chan, R. K., Lau, C. C., Tsao, S. W., Knapp, R. C., and Berkowitz, R. S. Molecular cloning of differentially expressed genes in human epithelial ovarian cancer. Gynecol. Oncol., *52*: 247-252, 1994.

5. Mok, S. C., Chan, W. Y., Wong, K. K., Cheung, K. K., Lau, C. C., Ng, S. W., Baldini, A., Colitti, C. V., Rock, C. O., and Berkowitz, R. S. DOC-2, a candidate tumor suppressor gene in human epithelial ovarian cancer. Oncogene *16*: 2381-2387, 1998.

6. Schummer, M., Ng, W. V., Bumgarner, R. E., Nelson, P. S., Schummer, B., Bednarski, D. W., Hassell, L., Baldwin, R. L., Karlan, B Y., and Hood, L. Comparative hybridization of an array of 21 500 ovarian cDNAs for the discovery of genes overexpressed in ovarian carcinomas. Gene *238*: 375-385, 1999.

7. Shayesteh, L., Lu, Y., Kuo, W.-L., Baldocchi, R., Godfrey, T., Collins, C., Pinkel, D., Powell, B., Mills, G. B., and Gray, J. W. *PIK3CA* is implicated as an oncogene in ovarian cancer. Nature Genet., *21*: 99-102, 1999.

8. Wang, K., Gan, L., Jeffery, E., Gayle, M., Gown, A. M., Skelly, M., Nelson, P. S., Ng, W. V., Schummer, M., Hood, L., and Mulligan, J. Monitoring gene expression

profile changes in ovarian carcinomas using cDNA microarray. Gene, *229*: 101-108, 1999.

9. Ingvarsson, S. The Brca1 and Brca2 proteins and tumor pathogenesis. Anticancer Res., *19*: 2853-2861, 1999.

10. Marks, J. R., Davidoff, A. M., Kerns, B. J., Humphrey, P. A., Pence, J. C., Dodge, R. K., Clarke-Pearson, D. L., Iglehart, J. D., Bast, R. C., Jr., and Berchuck, A. Overexpression and mutation of p53 in epithelial ovarian cancer. Cancer Res., *51*: 2979-29, 1991.

11. Randall, T. C., Bell, K. A., Rebane, B. A., Rubin, S. C., and Boyd, J. Germline mutations of the BRCA1 and BRCA2 genes in a breast and ovarian cancer patient. Gynecol. Oncol., *70*: 432-434, 1998.

12. Levine, A. J. p53, the cellular gatekeeper for growth and division. Cell, *88*: w323-331, 1997.

13. Scully, R., Chen, J., Plug, A., Xiao, Y., Weaver, D., Feunteun, J., Ashley, T., and Livingston, D. M. Association of BRCA1 with Rad51 in mitotic and meiotic cells. Cell, *88*: 265-275, 1997.

14. Sharan, S. K., Morimatsu, M., Albrecht, U., Lim, D. S., Regel, E., Dinh, C., Sands, A., Eichele, G., Hasty, P., and Bradley, A. Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking Brca2. Nature, *386*: 804-810, 1997.

15. Zhang, H., Somasundaram, K., Peng, Y., Tian, H., Zhang, H., Bi, D., Weber, B. L., and El-Deiry, W. S. BRCA1 physically associates with p53 and stimulates its transcriptional activity. Oncogene, *16*: 1713-1721, 1998.

16. Auersperg, N., Maines-Bandiera, S. L., Dyck H. G., and Kruk, P. A. Characterization of cultured human ovarian surface epithelial cells: phenotypic plasticity and premalignant changes. Lab. Invest., *71*: 510-518, 1994.

17. Baldwin, R. L., Yamada, S. D., Bristow, R. E., Chen, L., and Karlan, B. Y. Ovarian epithelial growth regulation. *In:* Sharp, F., Blackett, T., Berek, J., and Bast, R. (eds), Ovarian Cancer 5, pp. 99-107. Oxford, England: ISPS Medica Media, 1998.

18. Auersperg, N., Siemens, C. H., and Myrdal, S. E. Human ovarian surface epithelium in primary cultures. In Vitro, *20*: 743-755, 1984.

19. Yamada, S. D., Baldwin, R. L., and Karlan BY. Ovarian carcinoma cell cultures are resistant to TGF-β1 mediated growth inhibition despite expression of functional receptors. Gynecol. Oncol., *75*: 72-77, 1999.

20. Chang, D. D., and Denny, C. Isolating differentially expressed genes by representational difference analysis. *In*: P. D., Seibert (ed.), RT-PCR Methods for Gene Cloning and Analysis, pp. 193-202. Natick, MA: Eaton Publishing, 1998.

21. Chang, D. D., Park, N.-H., Denny, C., Nelson, S., and Pe, M. Characterization of transformation related genes in oral cancer cell lines by parallel analysis of differentially expressed gene fragments using arrayed filters. Oncogene, *16*: 1921-1930, 1998.

22. DeRisi, J., Penland, L., Brown, P. O., Bittner, M. L., Meltzer, P. S., Ray, M., Chen, Y., Su, Y. A., and Trent, J. M. Use of a cDNA microarray to analyse gene expression patterns in human cancer. Nature Genet., *14*: 457-460, 1996.

23. Kiechle-Schwarz, M., Bauknecht, T., Schmidt. J., Walz, L., and Pfleiderer, A. Recurrent cytogenetic aberrations in human ovarian carcinomas. Cancer Detect. Prevent., *19*: 234-243, 1995.

24. Thompson, F. H., Emerson, J., Alberts, D., Liu, Y., Guan, X. Y., Burgess, A., Fox, S., Taetle, R., Weinstein, R., Makar, R., Powell, D., and Trent, J. Clonal chromosome abnormalities in 54 cases of ovarian carcinoma. Cancer Genet. Cytogenet., *73*: 33-45, 1994.

25. Yang-Feng, T. L., Li, S., Han, H., Schwartz, P. E. Frequent loss of heterozygosity on chromosomes Xp and 13q in human ovarian cancer. Int. J. Cancer, *52*: 575-580, 1992.

26. Tapper, J., Sarantaus, L., Vahteristo, P., Nevanlinna, H., Hemmer, S., Seppala, M., Knuutila, S., and Butzow, R. Genetic changes in inherited and sporadic ovarian carcinomas by comparative genomic hybridization: extensive similarity except for a difference at chromosome 2q24-q32. Cancer Res., *58*: 2715-2719, 1998.

27. Fogh, J., and Trempe, G. *In*: J. Fogh (ed.), Human Tumor Cells In Vitro, pp. 115-154. New York, NY: Plenum Press, 1975.

28. van Haaften-Day, C., Russell, P., Rugg, C., Wills, E.J., and Tattersall, M. H. Flow cytometric and morphological studies of ovarian carcinoma cell lines and xenografts. Cancer Res., *43*: 3725-3731, 1983.

29. Ward, B. G., and Wallace, K. Localization of the monoclonal antibody HMFG2 after intravenous and intraperitoneal injection into nude mice bearing subcutaneous and intraperitoneal human ovarian cancer xenografts. Cancer Res., *47*: 4714-4718, 1987.

30. Wilson, A. P. Characterization of a cell line derived from the ascites of a patient with papillary serous cystadenocarcinoma of the ovary. JNCI, *72*: 513-521, 1984.

31. Watson, R. H., Neville, P. J., Roy, W. J., Jr., Hitchcock, A., and Campbell, I. G. Loss of heterozygosity on chromosomes 7p, 7q, 9p and 11q is an early event in ovarian tumorigenesis. Oncogene, *17*: 207-212, 1998.

32. Velculescu, V. E., Zhang, L., Voglestein, B., and Kinzler, K. W. Serial analysis of gene expression. Science, *270*: 484-487, 1995.

33. Horuchi, K., Amizuka, N., Takeshita, S., Takamatsu, H., Katsuura, M., Ozawa, H., Toyama, Y., Bonewald, L. F., and Kudo A. Identification and characterization of a novel protein, periostin, with restricted expression to periosteum and peridontal ligament and

increased expression by transforming growth factor β. J. Bone Miner. Res., *14*: 1239-1249, 1999.

34. Godwin, A. K., Testa, J. R., and Hamilton, T. C. The biology of ovarian cancer development. Cancer, *71*: 530-536, 1993.

35. RDG-CAP (βig-h3) enhances the spreading of chondrocytes and fibroblasts via integrin $\alpha_1\beta_1$. Biochim. Biophys. Acta, *1451*. 196-205, 1999.

36. Olsen, H. S., Cepeda, M. A., Zhang, Q. Q., Rosen, C. A., and Vozzolo, B. L. Human stanniocalcin: a possible hormonal regulator of mineral metabolism. Proc. Natl. Acad. Sci. USA, *93*: 1792-1796, 1996.

37. Hanna, E. A., Umhauer, S., Roshong, S. L., Piechocki, M. P., Fernstrom, M. J., Fanning, J. D., and Ruch, R. J. Gap junctional intercellular communication and connexin 43 expression in human ovarian surface epithelial cells and ovarian carcinomas in vivo and in vitro. Carcinogenesis, *20*: 1369-1373, 1999.

Table 1. Characteristics of ovarian epithelial cells used for comparative gene expression studies.

Culture Type	Pathologic Diagnosis	cytokeratin	vimentin	Factor VIII	p53	Karyotypes	Application
HOSE 231	normal	++	++	neg.	n/d	46:XX	cDNA-RDA
HOSE 250	normal	++	++	neg.	n/d	46:XX	"
CSOC 817	papillary serous	++	++	neg.	++	45:X[12]/46XX[18] (60% of cells had one X)	"
CSOC 826	papillary serous	++	++	neg.	++	47:XX,+mar[2]/46:XX[18] (10% +marker)	"
HOSE 224	normal	++	++	neg.	n/d	n/d	cDNA filter array hybridization
HOSE 246	normal	++	++	neg.	neg.	n/d	"
HOSE 253	normal	++	++	neg.	n/d	n/d	"
HOSE 254	normal	+	++	neg.	n/d	n/d	"
HOSE 256	normal	++	++	neg.	n/d	n/d	"
CSOC 823	papillary serous	++	++	neg.	neg.	n/d	"
CSOC 824	papillary serous	+	++	neg.	+	n/d	"
CSOC 827	papillary serous	++	++	neg.	neg.	n/d	"
CSOC 834	papillary serous	++	++	neg.	+	n/d	"
CSOC 839	papillary serous	+	++	neg.	neg.	n/d	"
CSOC 843	papillary serous	++	++	neg.	++/+++	n/d	"
CSOC 846	papillary serous	++	++	neg.	++	n/d	"
CSOC 869	papillary serous	+++	++	neg.	++	n/d	"
CSOC 871	papillary serous	+++	++	neg.	++	n/d	"
CSOC 872	papillary serous	+	++	neg.	+	n/d	"

Pathological diagnosis of the primary cultures used for cDNA-RDA or cDNA filter array hybridization experiments are shown. Immunohistochemical analysis was performed to ensure that cells had homogeneous cytokeratin and vimentin staining with no factor VIII staining. p53 immunostaining results are also presented. Increasing intensities of staining are indicated from "+" to "+++"; HOSE, human ovarian surface epithelial cells; CSOC, Cedar-Sinai malignant ovarian carcinomas.

Table 2. Identification of genes preferentially expressed in HOSE cells

2.5-3.0		>3.0	
Name	Accession number	Name	Accession number
		aldehyde dehydrogenase 1	K03000
adrenomedullin	D14874	**Connexin 43**	**M65188**
BAC 360 F12 Xq28	AC002523	hCOX-2	U04636
basic fibroblast growth factor	**M27968**	High sulphur keratins-B2A/B2D	X01610
DCRR1	D83327	**ICAM-1**	**J03132**
fumurase precursor	U59309	Il-6 inducible 26 kDa protein	M54894
PRAME- antigen of melanoma	**U65011**	**Insulin-like growth factor bp 5**	**L27560**
pyruvate dehydrogenase kinase	U54617	Keratin 19	Y00503
EST- cDNA clone	AA187731	**MASP**	**D28593**
EST- CD44	**N32466**	**MHC DNA**	**AB000879**
EST-gap junctional protein alpha 5/connexin 40	**AA436946**	nuclear pore complex-assoc. TPR	U69668
EST-HLA1 MHC27	**AA293071**	**Stanniocalcin**	**U25997**
EST-neuroligin	**AI093247**	EST-BAC clone 3p26-OXTR gene	AA235188
EST-retanoic acid induced 3 (RAI3)	AA112374	**EST- CD9**	**AA339020**
EST-regulator of G protein signalling (RGS4)	T74284	EST- CTX	AA406389
		EST-DVS27 related protein	AA428482
		EST-human clone 3930	AA776733
		EST-KIAA1199 protein	AA852453
		EST-MHC II alpha chain	**AA455820**
		EST-pregnancy specific beta 1 glycoprotein 6 (PSG6)	**AA772680**
EST	N42148	EST	AA425042
EST	AA425042	EST	AA569767
		EST	R18293
2 novel		3 novel	

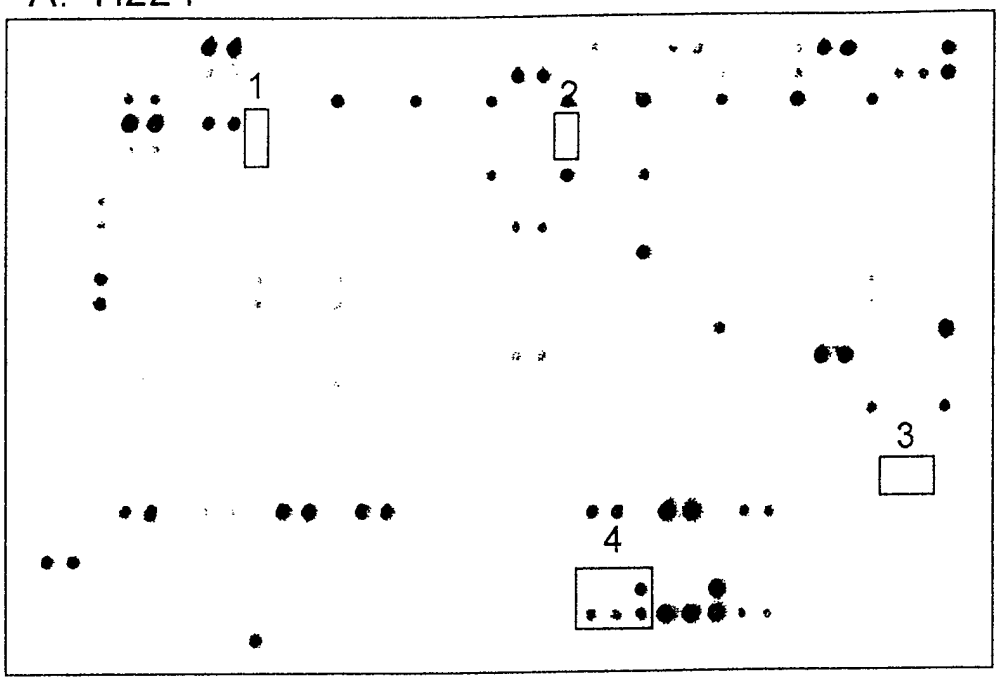
Genes preferentially expressed in HOSE cells with the GenBank matches are listed. Bold lettering indicates genes encoding proteins that are expressed on the cell membrane or that are secreted. Clones that failed to match any entry in the GenBank database (as of November 15, 1999) in the BLASTN searches are considered novel.

Table 3. Identification of genes preferentially expressed in CSOC cells

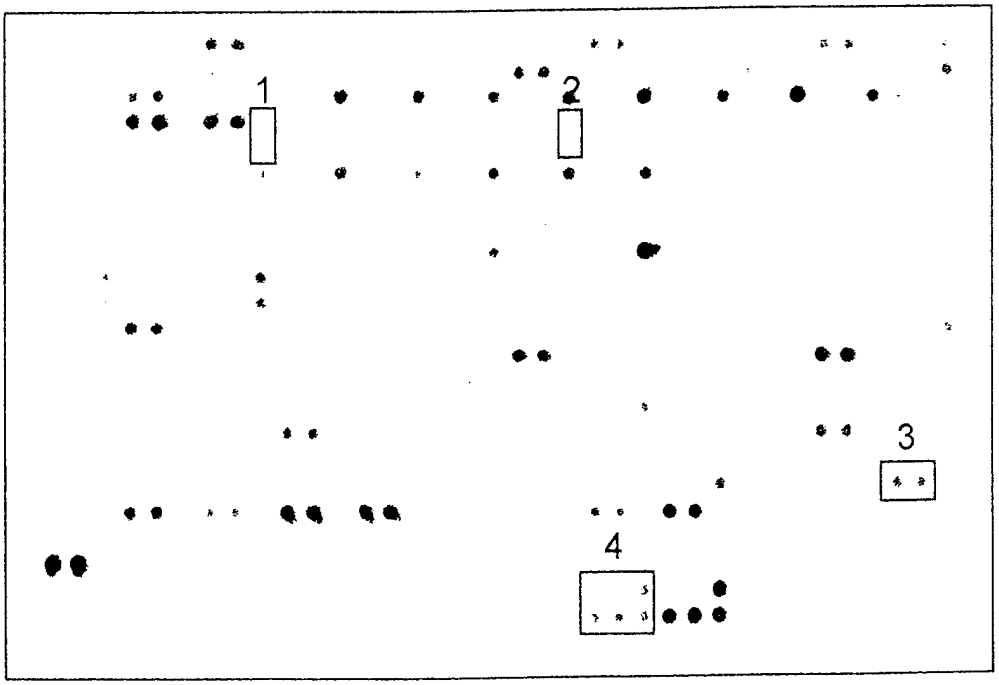
2.5-3.0		>3.0	
Name	Accession number	Name	Accession number
collagen alpha 1 type XV	**L25286**	**collagen alpha 2 type I**	**Z74616**
collagen alpha 3 type VI	**X52022**	**osteoblast specific factor-2**	**D13665**
cPLA2	M68874		
E1-E2 ATPase	AF011337	**EST- Annexin IV**	**W12985**
MEGF5	**AB011538**	**EST - Annexin VI**	**AA594856**
p37NB	U32907	EST- collagen c-prot enhancer	T49144
SPARC/osteonectin	**J03040**	**EST- collagen**	**Z33436**
EST	R67289	EST	W79345
1 novel			

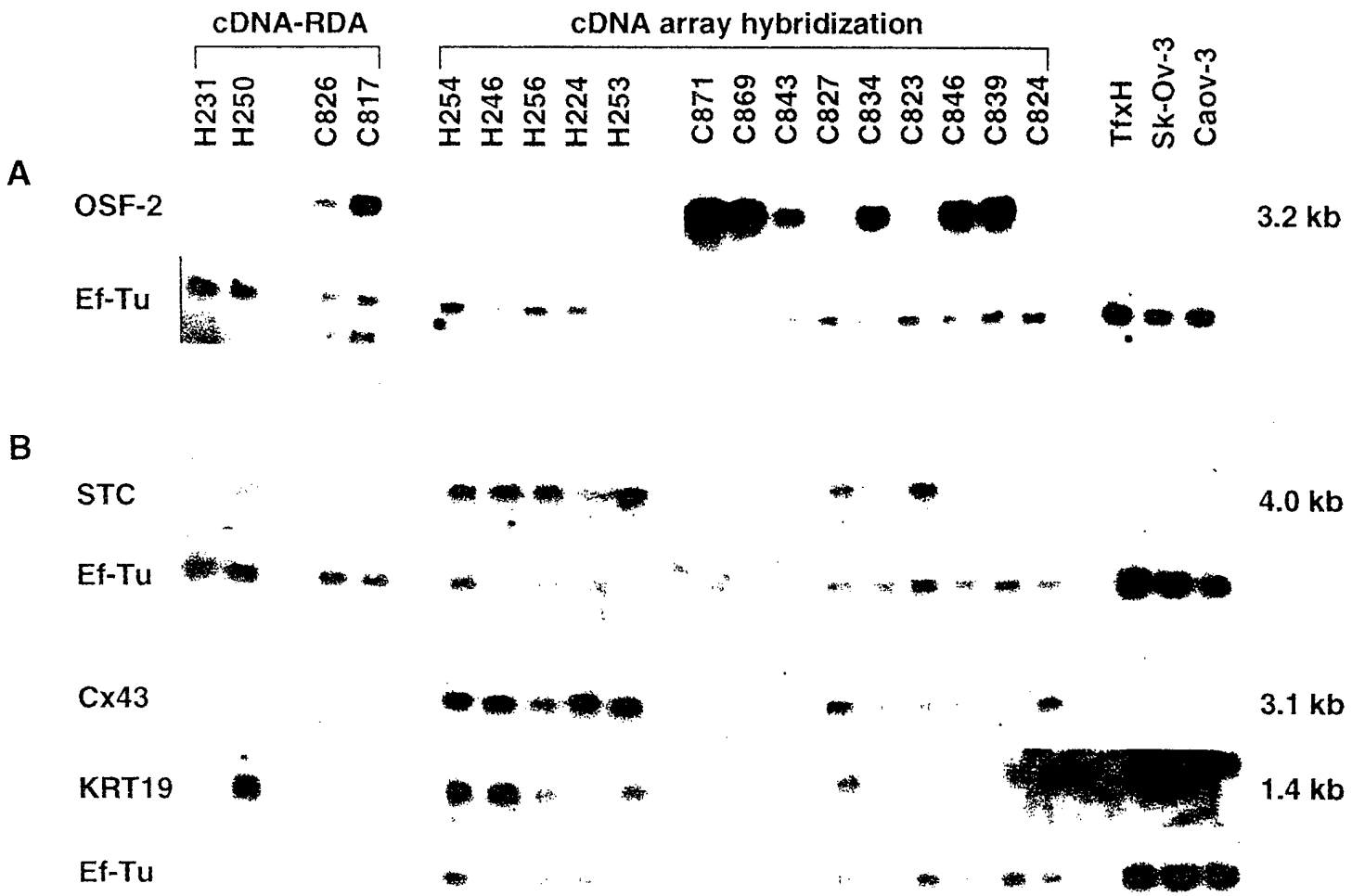
Genes preferentially expressed in CSOC cells with the GenBank matches are listed. Bold lettering indicates genes encoding proteins that are expressed on the cell membrane or that are secreted. Clones that failed to match any entry in the GenBank database (as of November 15, 1999) in the BLASTN searches are considered novel.

A. H224



B. C869




cDNA-RDA
cDNA array hybridization
H231
H250
C826
C817
H254
H246
H256
H224
H253
C871
C869
C843
C827
C834
C823
C846
C839
C824
TfxH
Sk-Ov-3
Caov-3
A
OSF-2
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B
STC
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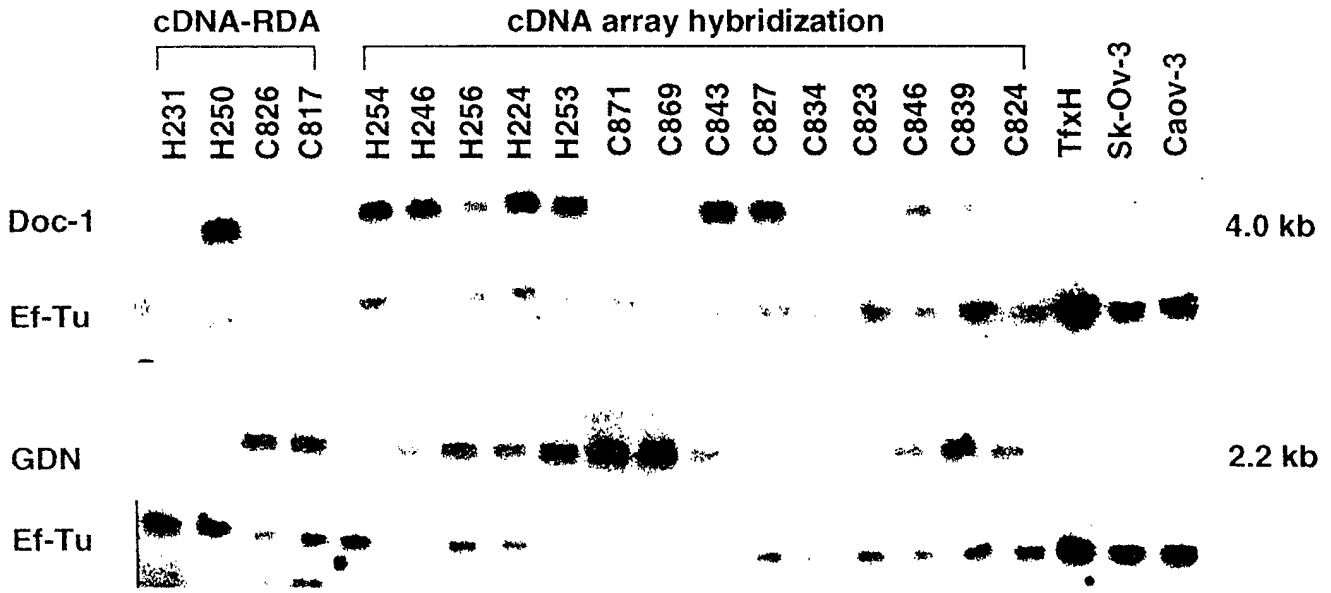
Cx43
3.1 kb
KRT19
1.4 kb
Ef-Tu


cDNA-RDA
cDNA array hybridization
H231
H250
C826
C817
H254
H246
H256
H224
H253
C871
C869
C843
C827
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C823
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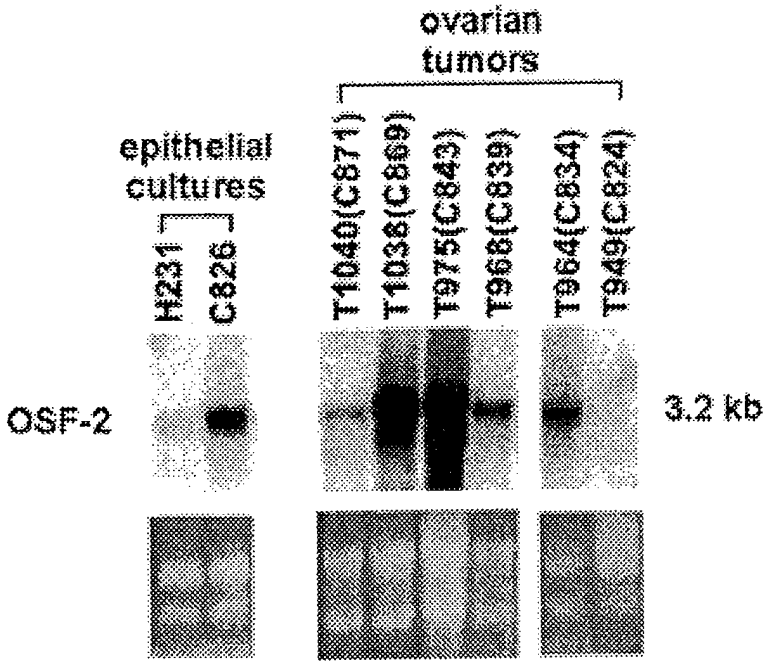
ovarian tumors
epithelial cultures
H231
C826
T1040(C871)
T1038(C869)
T975(C843)
T968(C839)
T964(C834)
T949(C824)
OSF-2
3.2 kb